V. THE EXAMINATION OF YEAST-FAT FOR THE PRESENCE OF VITAMINS A AND D BEFORE IRRADIATION AND OF VITAMIN D AFTER IRRADIATION.

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(*Received January 2nd, 1928.*)

In 1924, one of the writers (I.S.-M.) with E. M. Luce carried out some experiments on rats in which the effect of the addition of yeast-fat to a diet deficient in the fat-soluble vitamins was tested [1925]. At the time at which this work was undertaken it was not generally recognised that the fat-soluble vitamin factor contained two components. Although this view was originally brought forward in 1922 by McCollum, Simmonds, Becker and Shipley [1922], it was not till after the publication of a paper by Steenbock and Nelson [1924] that the presence of two distinct vitamins in cod-liver oil was clearly established; a deficiency of one of these (A) was shown to be responsible for the symptoms of ophthalmia and a deficiency of the other (D) was correlated with defective calcification of the bones, while both were able by their absence to limit growth. Since 1924, it has been clearly recognised that both vitamins A and D are present in cod-liver oil but that only the effect of the D vitamin can be replaced by irradiation of the animal. The results obtained by Luce and Smedley-MacLean showed that the addition of yeast-fat to a diet deficient in the fat-soluble vitamins resulted both in increased growth and in increased calcification of the bones; they attributed these results to the presence of the fat-soluble vitamin A in the yeast-fat since at that time the vitamins A and D were not differentiated. In the light of present knowledge the results then obtained would be interpreted as indicating the presence of vitamin D in the unirradiated yeast-fat and as yielding no information as to the presence or absence of vitamin A. The influence of yeast-fat in promoting growth confirmed the conclusions previously drawn by Hopkins [1912] from his experiments in which a marked effect on growth was obtained by the addition of the ether-soluble extract of yeast to a diet deficient in the fat-soluble vitamins. It seemed therefore of interest to repeat the work of Luce and Smedley-MacLean and to extend it to a separate examination for the two vitamins A and D which improved technique has now made possible.

Examination of yeast-fat for vitamin A.

The test for vitamin A was made by using the method described by Chick and Roscoe [1926]; young rats of about 40 g. weight were placed upon a diet[1] deficient in vitamins A and D and after about 5 weeks on this diet, the hardened cottonseed oil, forming about 10 % of it, was replaced by the same oil which had been irradiated for 30 minutes, using a mercury vapour quartz lamp, thus supplying vitamin D. The rats then began to grow more vigorously but ceased growing again in another two weeks and began to show signs of decline, with symptoms of xerophthalmia and general vitamin A deficiency. At this point 10 drops of yeast-fat, about 0·18 g., were administered to 2 rats and 5 drops to a third rat. The yeast-fat used was prepared by the direct extraction of pressed brewery yeast with ether, just as was the fat used by Luce and Smedley-MacLean in their experiments. The rats disliked it very much but took it fairly well. There was no restoration of growth but a progressive decline of condition with falling weight until the rats had to be killed after from 10 to 20 days of treatment with very severe symptoms of xerophthalmia and vitamin A deficiency (see Fig. 1).

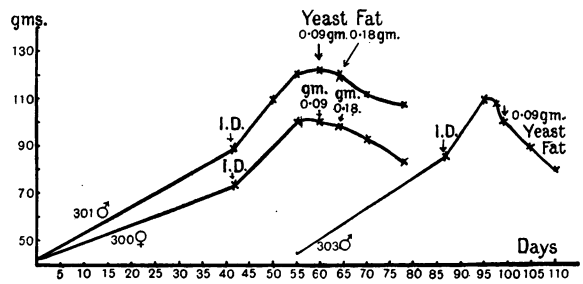

Fig. 1. *Vitamin A in total yeast-fat.* Three rats received a diet deficient in both vitamins A and D up to the point I.D. where a diet deficient in vitamin A only was substituted. When growth ceased a daily dose of 0·09 g. total yeast-fat was administered, which in the case of rats 300 and 301 was increased after several days to 0·18 g.

No resumption of growth occurred and the decline in weight and condition progressed until the rats showed severe symptoms of vitamin A deficiency, and had to be killed.

It would appear therefore that vitamin A is absent from yeast-fat or present in so small a quantity that it cannot be detected in such a dose as it was found possible to administer. The growth induced in rats in the experiment of Luce and Smedley-MacLean, when a daily dose of 0·14 g. fat was administered, prepared similarly by directly extracting dried pressed brewery yeast with ether, was probably therefore not due to the presence of vitamin A as was then supposed but would be accounted for if the fat contained vitamin D.

[1] The diet used was made up as follows: inactivated caseinogen, 300 g.; starch, 750 g.; hardened cottonseed oil, 225 g.; marmite, 75 g.; salt mixture, 75 g.; lemon juice, 75 cc.; distilled water, 900 cc. The salt mixture consisted of sodium chloride, 51·9 parts; magnesium sulphate, 164 parts; sodium dihydrogen phosphate, 104·1 parts; dipotassium hydrogen phosphate, 286·2 parts; calcium phosphate, 162 parts; calcium lactate, 390 parts; ferric citrate, 35·4 parts.

Examination of yeast-fat for vitamin D.

In testing for the presence of vitamin D, young rats of about 40 g. weight were placed upon the same diet, deficient only in vitamins A and D, as was used in making the test for vitamin A, but *without* the subsequent addition of any irradiated cottonseed oil. Sexes and litters were distributed as evenly as possible among the different experimental groups. After the rats had been for 5 weeks or more on the diet, and at a time when their weight curves were flat, the dose of yeast-fat was administered daily for a period of about 5 weeks, at the end of which time the rats were killed; the bones of both hind legs were then dissected out, dried, extracted with ether and alcohol and the percentage weight of ash determined and compared with the similar values obtained in the controls.

The growth, which takes place during and in response to the administration of the test dose of vitamin D, is taken as some indication of the potency of the given dose, particularly at the beginning of administration. The amount of this growth is, however, also dependent upon the amount of the rat's reserve of vitamin A and it cannot therefore be taken as an accurate criterion. It is, however, very helpful in adjusting the size of the dose in the first instance: if no growth response takes place in the first few days after the beginning of administration of the dose, it can be assumed that the dose is too small and it is then easy to increase it instead, perhaps, of carrying through a large experiment on a whole series of inadequate doses.

When carrying out the test for the detection of vitamin D, a considerable quantity of yeast-fat which had been prepared from yeast, incubated for 48 hours in the glucose-phosphate solution, as described on p. 30 (cp. Specimen I, p. 30), was available. As the supply of yeast-fat prepared by direct extraction of dried pressed brewery yeast which had been used for the examination for vitamin A was now exhausted, it was decided to carry out the tests for vitamin D on the fat derived from the incubated yeast, the sterol content of the two fats being almost identical (see Table I).

A dose of 10 drops (0·14 g.) of the fat was administered daily for some weeks to each of 2 rats but the results, both as regards growth and as regards percentage ash in the dried bone (Table II), were similar to those yielded by the controls, and must be regarded as entirely negative. The fat derived from the incubated yeast, although similar in sterol content, did not behave like the fat used in the previous experiments of Luce and Smedley-MacLean. In the incubated yeast there is a rapid and abnormal conversion of carbohydrate to fat and sterol. As will be seen later, after irradiation the fat obtained from the incubated yeast was as potent in vitamin D as the fat obtained directly from the pressed yeast freshly removed from the wort. Luce and Smedley-MacLean state that they had no evidence of the presence of the fat-soluble vitamin except in the fat derived from freshly pressed yeast as received from the brewery. This would lead to the important deduction

that some condition is present when yeast is growing in wort where a free supply of nitrogenous food is available which can produce the same result as irradiation, though to a much more limited extent. It is proposed to make a comparison of yeast-fat prepared by various methods and to ascertain what are the conditions of its preparation which lead to the presence in it of vitamin D.

Examination of irradiated yeast-fat for vitamin D.

Since Rosenheim and Webster [1927] have identified the provitamin D as ergosterol itself or as some closely related substance, the investigation of irradiated yeast-fat, which contains this sterol in relatively large amount, is of special interest.

Three different specimens of yeast-fat were used for irradiation; the methods of preparation and characteristic properties of these were as follows.

Specimen I. This was prepared from pressed brewery yeast which had been incubated for 48 hours in a well-aerated solution containing 5 % glucose, 0·37 % Na_2HPO_4 and 0·029 % KH_2PO_4 [Smedley-MacLean and Hoffert, 1924]. The yeast was allowed to settle, filtered off and repeatedly extracted with cold alcohol. The solvent was removed under diminished pressure, the residue dissolved in ether and as the lipins were needed for other experiments, they were removed by precipitation with acetone, and the acetone-soluble fat used for irradiation.

Specimen II. This was obtained from the original pressed yeast by repeatedly extracting it with alcohol at the ordinary temperature. It differed from Specimen I in that the yeast had not been incubated in the glucose-phosphate solution before its extraction. Starting with the same quantity of pressed yeast, the fat content is never more than one-fifth of that obtained from yeast which has been incubated. The lipin fraction was not removed from this specimen.

Specimen III. This was obtained from another sample of yeast which had been first incubated in the glucose-phosphate solution as in Specimen I. The yeast was then boiled with normal HCl for 2 hours, filtered and the dried residue extracted with ether in a Soxhlet apparatus.

The properties of these three specimens are shown in Table I. The sterol content was estimated by precipitation with digitonin after hydrolysis of the fat.

Table I. *Properties of specimens of yeast-fat.*

	Iodine value of fat	Unsapon. fraction %	Sterol %	Iodine value of unsapon. fraction
Specimen I	146·5	41·7	9·7	271·4
„ II	137·6	35·2	10·2	285·3
„ III	156·5	31·3	2·65	205·4

Method of irradiation.

A drop of yeast-fat was weighed on a Petri dish after being spread over as large a surface as possible and exposed for 1 hour to the rays from a mercury vapour quartz lamp at a distance of 32 cm.

Estimation of dose.

Having previously determined the sterol content of the fat, the weight of fat which would contain 1/400 mg. of sterol was taken as unit dose and the number of unit doses contained in the drop of fat calculated. Fresh fat was irradiated each day. The fat was then dissolved in glycerol, the quantity of glycerol added being such that one drop of the glycerol solution of fat would contain the unit dose of sterol.

In one experiment the unit dose was reduced to one-fifth of that originally taken by further dilution with glycerol. In this case the dose of sterol actually given was 1/2000 mg. Controls and rats receiving less than the maximum dose of fat and glycerol received corresponding amounts of pure glycerol only. The work of Rosenheim and Webster [1927] has shown that ergosterol after irradiation with ultra-violet light is extraordinarily potent in preventing the onset of rachitic symptoms in rats fed on a diet deficient in the fat-soluble vitamins, the minimum dose of the irradiated sterol necessary being from 1/10,000 to 1/20,000 mg. or even less. In estimating the dose of fat which would be equivalent to this dose of activated sterol, two facts have to be borne in mind.

(1) Yeast contains two sterols, both of which are precipitated by digitonin [Smedley-MacLean, 1928]. One of these, ergosterol, becomes active when irradiated: the biological behaviour of the other, zymosterol, is at present under investigation. The amount of sterol in yeast, as estimated by precipitation with digitonin, does not therefore represent the amount of ergosterol present.

(2) So far experiments have not been carried out to determine the most favourable conditions for irradiation. It is possible that a product of greater activity might be obtained by irradiating the fat in solution in a suitable organic solvent. In the case of the sterols irradiation of the solids does not give satisfactory results and it is quite possible that the irradiation of the highly pigmented yeast-fat is not as satisfactory as irradiation in a suitable solvent, a point that requires further investigation.

Biological tests with irradiated yeast-fat.

The biological test for vitamin D was carried out on the irradiated fat in the same manner as that on the non-irradiated fat described above (p. 29).

Specimen I. Doses of 0·005, 0·025 and 0·125 mg. of the fat were administered to groups of 3 rats each and the results compared with those given by controls (see Tables II and III). The two larger doses gave results which must be regarded as the same; both of them show a very high percentage ash. The dose of 0·005 mg. produced some increase in ash content over that shown by controls of the same sex and litter, but the controls are themselves rather high and a dose of 0·005 mg. would appear to be approaching the minimum dose of this particular sample of irradiated yeast-fat.

Table II.

A. *Showing percentage ash in the dried extracted bones of rats receiving various doses of yeast-fat added to a diet deficient in fat-soluble vitamins.*

			Irradiated fat						
			Specimen I. From incubated yeast: acetone-soluble fraction. Sterol = 9·7 %			Specimen II. From yeast direct from brewery. Sterol = 10·2 %		Specimen III. From incubated yeast hydrolysed Sterol = 2·6 %	Unirradiated fat, from incubated yeast
Litter	Sex	Control							
Dose of fat in mg. ...			0·005	0·025	0·125	0·025	0·125	0·125	140
Dose of sterol (mg.) ...			$\frac{1}{2000}$	$\frac{1}{400}$	$\frac{1}{80}$	$\frac{1}{400}$	$\frac{1}{80}$	$\frac{1}{300}$	—
649	♀	—	—	60·0	57·3	—	—	—	—
657	♂	50·0	—	55·3	57·9*	53·1	56·0	—	46·5
657	♀	46·8	—	57·1	56·9	53·6	56·7	—	48·5
649	♂	54·6	54·9	—	—	—	—	55·1	—
649	♂	53·0	—	—	—	—	—	55·0	—
648	♂	53·1	55·7	—	—	—	—	—	—
648	♂	55·8	57·1	—	—	—	—	—	—
648	♀	54·7	—	—	—	57·1	58·9	57·7	—

B. *Showing total increase in weight (g.) during first three weeks of treatment of the same series of rats with various doses of yeast-fat.*

649	♀	—	—	24	41	—	—	—	—
657	♂	3	—	35	46	19	35	—	6
657	♀	10	—	43	56	20	38	—	3
649	♂	18	17	—	—	—	—	0	—
649	♂	20	—	—	—	—	—	22	—
648	♂	3	23	—	—	—	—	—	—
648	♂	12	19	—	—	—	—	—	—
648	♀	10	—	—	—	22	25	20	—

* Value slightly too high, being derived from one heating of the bone only.

Specimen II. Doses of 0·025 and 0·125 mg. were given to groups of 3 rats. Some of the rats in these groups were also litter mates of those receiving corresponding doses of Specimen I, and where this is the case, the results are directly comparable. This irradiated fat does not appear to be quite as rich in vitamin D as is Specimen I. The dose of 0·025 mg. shows a considerably higher percentage ash than that yielded by the corresponding controls, but not as high as that yielded by the dose of 0·025 mg. of Specimen I.

Specimen III. A dose of 0·125 mg. of this fat was given to 3 rats. In each case the percentage ash is somewhat raised but the results are too fragmentary to draw quantitative conclusions from them. Specimen III was found to have a very low sterol content and there are indications that when irradiated it has less activity than Specimens I and II, whose sterol contents are approximately the same, but of which Specimen I appears to be slightly more active when irradiated. It has already been pointed out [Smedley-MacLean, 1928] that it is not yet known whether both the highly unsaturated sterols in yeast-fat are capable of yielding vitamin D on irradiation or

whether only the ergosterol is so changed. If the latter be the case, the vitamin D content of the fat after irradiation would not bear any constant relation to the total sterol content of the fat.

SUMMARY.

1. Yeast-fat was examined for its content of vitamin A and of vitamin D, the latter both before and after irradiation of the fat.

2. A sample of total fat, derived from pressed brewer's yeast, showed no activity for vitamin A, when a daily dose of 0·18 g. was given to rats on a diet deficient only in vitamin A.

3. A sample of acetone-soluble fat, prepared from yeast which had been incubated in a carbohydrate-phosphate solution, was found neither to promote the growth nor the bone formation of rats on a diet deficient only in fat-soluble vitamins, when a dose of 0·14 g. was given daily. It was therefore concluded that such a sample of yeast-fat was devoid of vitamin D. Luce and Smedley-MacLean had previously found that a sample of total yeast-fat, derived directly from pressed brewer's yeast, did promote growth and calcification of rats. The explanation of the discrepancy probably lies in the different treatment of the yeast from which the specimens of fat were obtained.

4. The same two kinds of yeast-fat, together with a sample of fat prepared from yeast after a preliminary boiling with normal acid, were irradiated with the mercury vapour quartz lamp and fed to rats on a diet deficient in fat-soluble vitamins, in very small daily doses. The best results were given by the fat derived from incubated yeast, which promoted growth and bone formation in a daily dose of 0·005 mg. The quantitative relationship of the vitamin D in the irradiated fat to the two sterols, ergosterol and zymosterol, present in the fat before irradiation is not yet worked out.

The writers desire to acknowledge their indebtedness to the Medical Research Council and to the Department of Scientific and Industrial Research respectively for grants which have made it possible for them to carry out this work.

REFERENCES.

Chick and Roscoe (1926). *Biochem. J.* **20**, 633.
Hopkins (1912). *J. Physiol.* **44**, 425.
Luce and Smedley-MacLean (1925). *Biochem. J.* **19**, 47.
McCollum, Simmonds, Becker and Shipley (1922). *J. Biol. Chem.* **53**, 293.
Rosenheim and Webster (1927). *Lancet*, **i**, 622.
Smedley-MacLean (1928). *Biochem. J.* **22**, 22.
Smedley-MacLean and Hoffert (1924). *Biochem. J.* **18**, 1273.
Steenbock and Nelson (1924). *J. Biol. Chem.* **56**, 192.